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(54) Title: COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW ASSAYS UTILIZING SOLUBLE SUBMICRON PARTICLES

(57) Abstract

The present invention discloses a simplified, sensitive and specific immunoassay test procedure for the determination and detection of an immunologically reactive analyte in aqueous sample. A capture membrane comprising a dendrimer which aligns a capture antibody on a solid surface so that the capture capability of the capture antibody is optimized, resulting in a minimum loss in binding activity between the capture antibody and the ligand.

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COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW ASSAYS UTILIZING SOLUBLE SUBMICRON PARTICLES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention discloses a dendrimer-based, lateral flow, colorimetric assay test system.

2. Description of the Prior Art

The past twenty years have seen significant advances in rapid immunodiagnostic techniques. These methods include radio-imunoassay, counter electrophoresis, ELISA and membrane strip tests utilizing reagents tagged with a colorimetric label (e.g. gold, colored latex, etc.). Rapid diagnostic tests utilizing gold labeled antibody to visualize the test results on antibody coated membranes have evolved to be the test—of—choice in cases where manual, fast, and inexpensive, non—instrument test formats are required. The major area of concern, when utilizing the rapid gold test format, is the relative lack of sensitivity when compared to tests such as ELISA, which utilize enzyme enhanced reactions and prolonged multiple incubations steps and prolonged time.

Over the years, there have been many gold immunoassay test systems developed to show positive results in the face of particular antigens or antibodies. These assay systems utilize a colloidal gold particle concentration immunoassay to achieve sensitive and selective detection of biological materials. Antibodies specific to the agent of interest are conjugated to colloidal gold particles. Colloidal gold consists of discrete, electron-dense, red—colored particles ranging from 10 nm to 100 nm in diameter with a very high extinction coefficient. When concentrated on solid surfaces, these particles can be visually observed. Labeled antibodies can be easily dried and reconstituted without losing activity or specificity.

If antigen is present in a collected sample, an immune complex will form between the colloidal gold-labeled detector antibody (Ab) and the antigen (Ag). The test sample is used to reconstitute a dried colloidal gold labeled antibody and the resulting mixture (antibody and test antigen) ascends chromatographically up a strip that has been layered with a capture antibody, or directly through an antibody coated

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membrane. The presence of a red stripe or a dot is indicative of a positive test. The test strips contain a positive control to ascertain that the test is working properly.

U.S. Patent No. 5,514,602 (Brooks, Jr. et al.) discloses a method of producing a metal sol reagent containing colloidal metal particles. A metal containing solution is reduced under optimized pH conditions to produce metal sol particles of a preselected size. The particles are coated with a coupling compound, and then bound with at least one selected immunochemically reactive component. Particles having different immunochemical specificities are also mixed to produce reagents having multiple selected immunochemical specificities.

U.S. Patent No. 5,384,265 (Kidwell et al.) discloses contacting a sample which may contain an analyte with a biomolecule which is bound to a catalytically active colloidal metal particles, to obtain an analyte-biomolecule—colloidal metal particle complex, separating the analyte—biomolecule—colloidal metal particle complex from the sample, reacting the analyte—biomolecule—colloidal metal particle complex with hydrazine in the presence of lucigenin at a pH of 8 to 11; and detecting light generated by the reaction of the analyte—biomolecule—colloidal metal particle complex in the presence of lucigenin.

U.S. Patent No. 5,294,369 (Shigekawa et al.) discloses a gold sol coated with alkanethiols and alkanethiol derivatives which provide groups on the sol available for the linking of binding moieties such as antibodies, antigens or ligands to the gold sol.

U.S. Patent No. 5,334,538 (Parker et al.) discloses a gold sol immunoassay system and device. The gold sol bead is held in a funnel member. Antibodies are associated with the gold sol bead. When the sample contacts the gold sol, it dissolves the bead. A second antibody is impregnated on an immunosorbent surface. When the dissolved gold sol passes this surface, any antigen already reacted with the first antibody present reacts with the second antibody forming a gold:first antibody:antigen:second antibody: immunosorbent complex. The gold sol acts as the visible label.

U.S. Patent No. 5,120,643 (Ching et al.) discloses a process for immunochromatography with colloidal particles. The method comprises the steps of: contacting a chromatographic medium with the test sample, with the medium comprising at least two reaction sites. The first reaction site comprises a dried solution of a labeled specific binding reagent in the presence of a meta-soluble protein, and a

second reaction site comprising an immobilized specific binding reagent in relation to the presence or amount of the analyte in the test sample. The labeled reagent is solubilized and at least a portion of the labeled reagent is transported to the second reaction site, with the binding dependent upon the presence or amount of the analyte in the test sample. The labeled reagent is detected at the second site to determine the presence or amount of the analyte in the test sample.

U.S. Patent No. 5,079,172 (Hari et al.) discloses a method and kit for detecting the presence of antibodies using gold-labeled antibodies. Microspheres coated with an antigen reactive with the first antibody are reacted with the first antibody from serum or other sources. The gold-labeled antibody is reacted with the first antibody antigen complex on the microsphere and detected. Preferably, the gold particles are detected using an electron microscope.

PCT / US95 / 04547 describes the use of soluble submicron particles (dendrimers) that are labeled with antibodies to coat membranes. The assay procedures described are flow-through ELISA and fluorescence immunoassays requiring washing steps in order to obtain a response.

Currently, most lateral flow and flow-through gold immunoassays utilize antibody bound directly to porous membranes or to particles such as glass or latex to capture antigen-antibody-gold colored complexes.

The original tests developed by New Horizons Diagnostics utilized primarily 20 nm gold particles to tag the antibodies. This resulted in a sensitivity level of about 50 ng for botulism toxin and around 10^5 organisms/cc for the quantity of specific bacteria that could be detected. Particles, 20 nm in diameter, were used initially because of the stability of the gold tagged antibody conjugate and the low background signal (if any) it gave in negative test samples. Ideally, it is better to label with a much larger gold colloid (anywhere between 50 nm to 100 nm) to increase the sensitivity of test results. However, the problem with using larger colloidal gold complexes for tagging has been the increase in non-specificity, which creates false positive reactions.

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SUMMARY OF THE INVENTION

The present invention proposes a much more sensitive immunoassay test, which is easier to use and interpret. The entire test is conducted on a test strip and the detection antibody is preferably a FAB fragment that has been labeled with a 50-100nm gold particle and immobilized on a test pad. The capture antibody is bound to a soluble submicron particle (dendrimer) which is used to coat the membrane and become the capture site. The invention provides a simplified, sensitive and specific test procedure for the determination and detection of an immunologically reactive analyte in an aqueous sample.

Specifically, the present invention discloses the use of capture dendrimers to align and secure capture antibodies on a solid surface so that the immunological activity of the capture antibody is not sterically hindered. Consequently, optimal binding capacity is achieved, resulting in a minimum loss in binding activity between the capture antibody and the ligand.

In another preferred embodiment of the invention, the gold immunoassay test system uses larger gold colloids for tagging antibodies (anywhere between 50 nm to 100 nm) to increase the sensitivity of test results, (i.e. it is easier to detect larger particles because of the color intensity) without any residual non-specific activity. The elimination of non-specific background activity problems results from the use of blocking chemistries which inhibit non—specific reactions without altering the desired specific-reaction, the use of dendrimer bound antibody as the capture reagent, and the use of specifically designed recombinant FAB antibodies.

FAB antibodies are unlike whole antibodies in that their FC or constant regions are eliminated. It is this region of the antibody molecule that often causes problems with non—specificity.

In this invention, the FAB antibodies for detecting a positive result are attached to gold colloidal masses, in the range of 50-100 nm. These gold-FAB antibody complexes are positioned on a test strip, downstream from where the antigenic sample is applied.

Additionally, other metals or dyes may be attached to the FAB antibodies in place of the gold particles.

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Further downstream from both the antigenic or ligand sample and the gold—FAB sample is a set of antibodies, specific for the gold-FAB antibody-antigen or antibody-ligand complex. These antibodies serve to concentrate the complex in one location, thereby allowing for a red stripe to appear on a set section of the test strip when there is a positive reaction.

The capture antibodies are located further downstream from the reagent and the sample site.

Dendrimers are one way of attaching the capture antibodies to the test strip. Dendrimers are three dimensional, tree-like polymers. The dendrimers have a small size, good solubility, high segmental density, interior void space, and low viscosity. Dendritic polymers can be constructed by both divergent and convergent synthetic methods. The divergent synthesis starts from a center core, and then grows each layer in a stepwise fashion, while the convergent method assembles exterior end groups and dendrons first before being coupled onto a core. Each re-iteration or layer is defined as a generation. The more layers there are in the dendrimeric structure, the more rigid the dendrimer molecule itself becomes. Up to about G8, a dense packing stage also occurs, where beyond this point, monomers can no longer react with every surface group quantitatively due to the stearic effect. In the case of immunoassays, the rigid, spherical protein—like sizes control the antibody binding direction, while the exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion onto a membrane surface. This property allows the dendrimer-antibody conjugate to self-assemble to its best binding orientation. As a result, the sensitivity and reproducibility of the immunoassay in the detection of different agents has been significantly improved, while the assay time has been shortened. In addition due to the nature of its low viscosity and self assembling capability of the dendrimer-antibody conjugates, the process for producing these tickets has been simplified, and has eliminated lot to lot variability.

Dendrimeters have the advantage that they can be synthesized with an exact uniform molecular weight, whereas conventional polymers always have a particular molecular weight distribution. In addition, dendrimers with particular functional groups can be manufactured with a defined number of such reactive groups.

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The coupling of antibodies to outer surfaces of dendrimers (for example polyamidoamine [PAMAMS] dendrimers) can be done by various well known chemistries which describe carbon sulfur, carbon-oxygen, and carbon-nitrogen coupling procedures. The exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion onto the surface of a membrane. The structural composition of the dendrimer controls the spatial arrangement of the attached antibody molecules. This assures the optimal binding activity of the immobilized capture antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and advantages of the present invention will become more readily apparent from the following description, reference being made to the accompanying drawings in which:

FIG. 1 is an overall view of the immunoassay test system;

FIG. 2 is an exploded view of the immunoassay test system;

FIG. 3 is a side view of the immunoassay test strip;

FIG. 4 is an overhead view of the immunoassay test strip;

Fig. 5 is a schematic drawing of the antigen-antibody reaction.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Referring to Figures 1—5, the immunoassay test system 1 comprises an enclosure 2, which is preferably plastic. This plastic enclosure comprises a top section 3 and a bottom section 4 which are held together by male 5 and female 6 peg joints.

The top section 3 of the enclosure 2 has an opening 7 for placing a sample. There is also an opening 8 to visualize the test results 9 and the control results 10.

The bottom section 4 comprises a tray 11 into which fits a test trip 12. The test strip 12 preferably has a membrane support 13. The membrane support 13 may be comprised of plastic, cardboard, or any other rigid material. On top of the membrane support 13 is a testing layer 14, preferably made out of nitrocellulose. On top of the nitrocellulose or testing layer 14 are the areas to which the appropriate reagents or samples are applied or affixed. The nitrocellulose/testing layer is affixed to the membrane support 13 by an adhesive 31.

At one end of the test strip 12 is the sample site 15 to which the sample is to be applied. This sample site 15 may have another nitrocellulose or adsorbent pad 23 residing on top of the testing layer, to which the sample is transferred. The sample may be in the form of an antigen or ligand 16 in a fluid.

The list of target ligands and anti-ligands which potentially may be detected includes antigens and ligands as well as antigens associated with bacteria, parasites, fungi, viruses, toxins, etc. Moreover, therapeutic drugs and controlled substances having small molecules, such as, for example, theophylline, may be detected or determined using the present invention.

The sample travels downstream from the sample site 15 to the gold immunoassay site 18 where LAB antibody-coated gold sol particles 19 reside. The gold particles 19 attached to the FAB antibodies 20 are preferably larger than 20 nm, more preferably in the range of about 50 to 100 nm, and most preferably in the range of from about 70 to 90 nm. Larger particles may also be used wherein a number of FAB antibodies 20 are attached to the gold particle 19. The gold sol labeled FAB antibodies 21 are preferrably dried and deposited on the strip 12.

The metal sol particles to be used in accordance with the present invention may be prepared by coupling an immunologically reactive substance directly to the gold particle. Additionally, the labeled component may be prepared by coupling the substance to the particle using a biotin/avidin linkage. In this latter regard, the substance may be biotinylated and the metal containing particle coated with an avidin compound. The biotin on the substance may then be reacted with the avidin compound on the particle to couple the substance and the particle together. In another alternative form of the invention, the labeled component may be prepared by coupling the substance, such as bovine serum albumin (BSA), and using this antibody as a carrier to bind to the metal particle.

The metal sol particles to be used in accordance with the present invention may be prepared by methodology which is well known. For instance, the preparation of gold sol particles is disclosed in an article by G. Frens, *Nature*, 241, 20-22 (1973). Additionally, the metal sol particles may be metal or metal compounds or polymer nuclei coated with metals or metal compounds, as described in U.S. Pat No. 4,313,734. Other methods well known in the art may be used to attach the gold particles to the FAB

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antibodies. The metal sol particles may be made of platinum, gold, silver, or copper or any number of metal compounds which exhibit characteristic colors and high extinction coefficients.

Similarly, the antibodies do not necessarily have to be attached to a metal sol particle, but may instead be attached to a dye with an extinction coefficient equal to or greater than gold.

There are a number of ways in which the gold labeled FAB antibodies 21 may be deposited on the strip 12. The gold labeled FAB antibodies 21 may be lyophilized or dried on the strip.

In the preferred embodiment, the gold labeled FAB antibodies are deposited and dried on a rectangular adsorbent pad 22, preferably about .25" x .25" or less. This FAB antibody pad 22 is positioned downstream from where the sample is applied on the strip 12. Preferably, the FAB antibody pad 22 fits underneath the distal end 24 of the sample pad 23.

If the sample contains an antigen or ligand 16 to which the gold FAB antibodies 21 react, there is an antigen-antibody bonding between the sample and the gold FAB antibodies 21. The antigen-gold FAB antibody complex 25 continues to migrate along the nitrocellulose strip 12 to the capture site 26 where the dendrimer bound antibody is fixed 12.

The antibodies 27 supported by the dendrimers 32 are designed to react specifically with the antigen, effectively forming an antibody-antigen-gold FAB antibody sandwich 29 if there is a positive reaction. If there is a negative reaction, no "sandwich" is formed, and the unreacted labeled antibody proceeds to the end of the strip 12 wherein an absorbent pad 30 absorbs the fluid and unreacted ligand that has migrated to the end of the strip 12.

To prepare the capture site 26, the antibodies are chemically bound to the dendrimers prior to their placement on the strip 12. This is then layered on the nitrocellulose strip and dried.

In performing the testing of an antigen or ligand, it should be noted that if there is a positive test result, the antigens or liqands will attach to the gold FAB antibodies as they migrate from the sample site, whereupon the antigens or ligands will attach to the

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antibodies attached to the dendrimers. At this point, the concentrated gold particles appear as a red to purple line. If, however, the ligands do not attach to the gold labeled antibodies, the antibodies will not be bound by the capture site 26.

The superior sensitivity of this test format allows for detection of antigens or ligands in picogram quantities.

Additionally, this test can also have a positive control line. The positive control line has an anti-Fab substance or antibody laid down at the appropriate spot on the strip, downstream from the sample test site and from the FAB reagent site. This line should always appear when FAB antibodies are used in the test. If the positive control is negative, then the test is invalid.

Most immunoassay procedures are conducted at essentially neutral pH (7.5 to 8.0). In the preferred embodiment of this invention, the pH is elevated from pH 9 to pH 12. This is necessary to ensure that the positive surface charge on the dendrimer—antibody complex does not react non—specifically with the FAB-Gold labeled antibody and therefore yield false positive results.

EXAMPLES:

LAB antibody specific to Botulism toxin A at a concentration of 1.45 mg/cc was labeled with colloidal gold approximately 70 nm in size, at pH 9.5 at a final concentration of 10 μg/ml of 70nm gold. The adsorption was completed overnight and the gold antibody conjugate was centrifuged removing any unlabeled antibody.

The gold FAB complex was placed in borate buffer with 0.1% non-IDET detergent and 15 μ1 was spotted onto a precut polyester membrane and dried in the oven at 45° c for one hour.

FAB antibody (anti Bot. tox) was chemically bound to polyamidoamine dendrimers at a concentration of about one antibody molecule per branched dendrimer site. The soluble dendrimer antibody conjugate was used to stripe a nitrocellulose chromatography strip.

Goat anti-Mouse FAB fragment was used as a positive control and it was striped in an area in close proximity to the test zone.

Another test strip was prepared as above with the following difference: (1) the gold labeled antibody was tagged with 20 nm gold. (2) the FAR anti-Bot. tox was

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layered directly onto the nitro cellulose membrane. Dilutions were made of Botulism toxin A. The antigen was placed in 200 μ1 of a potassium carbonate solution, pH 11.0. The test solution was dispensed into the well of a test device described herein to which the test strip was added.

RESULTS	200 ng	100 ng	50 ng	10 ng	1 ng	0.5 ng
70 nm gold-antibody-dendrimer Capture	+	+	+	+	+	+
20 nm gold-straight capture	+	+	+	—	—	—

The buffers and BSA were run as negative controls. There were no false positive reactions.

Many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood within the scope of the appended claims that the invention may be protected otherwise than as specifically described.

What is claimed is:

1) A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a testing layer, said testing layer comprised of an absorbent material;

a sample site at one end of the testing layer, to which a ligand is applied;

a reagent—colorimetric imnunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of antibodies attached to colorimetric particles thereby forming colorimetric labeled antibodies, said colorimetric labeled antibodies residing at said reagent—colorimetric immunoassay site;

a capture site, said capture site comprising dendrimers and capture antibody, forming dendrimer-capture antibody complexes to retain ligand and ligand-colorimetric labeled antibody complexes;

wherein said ligand, when placed on said sample site, migrates to the reagent—colorimetric-immunoassay site, and then migrates to the capture site comprising said dendrimers, such that if said ligand reacts positively with the colorimetric labeled antibodies, said ligand—colorimetric labeled antibody complexes migrate to the capture site, and a positive response will appear as a colorimetric line at the capture site when a capture antibody— ligand—colorimetric labeled antibody sandwich is formed.

2) The immunoassay test system of claim 1, wherein said antibodies at said reagent-colorimerric immunoassay site are FAB antibodies.

3) The immunoassay test system of claim 1, wherein said colorimetric particles are selected from the group consisting of gold sol particles, silver particles, platinum particles, copper particles and encapsulated dyes.

4) The immunoassay test system of claim 3, wherein the colorimetric particles are gold sol particles.

5) The immunoassay test system of claim 4, wherein said gold sol particles may range in size from about 20 nm to about 100 nm.

6) The immunoassay test system of claim 5, wherein said gold sol particles may range in size from about 50 nm to about 100 nm.

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7) The immunoassay test system of claim 1, wherein said antibodies at the reagent-colorimetric immunoassay site are FAB antibodies.

8) The immunoassay test system of claim 1, wherein said ligand is an antigen.

9) The immunoassay test system of claim 1, wherein the ligands and anti-ligands which may be detected are selected from the group consisting of antigens and ligands associated with bacteria, parasites, fungi, viruses, toxins, therapeutic drugs, and controlled substances having small molecules.

10) The immunoassay test system of claim 1, wherein said dendrimers for aligning said capture antibodies are starburst dendrimers.

11) The immunoassay test system of claim 1 wherein the test is performed at a pH between about 8 to about pH 12.

12) The immunoassay test system of claim 11, where the test is performed is at a pH betweeb pH 9.5 to about pH 11.5

Fig 1

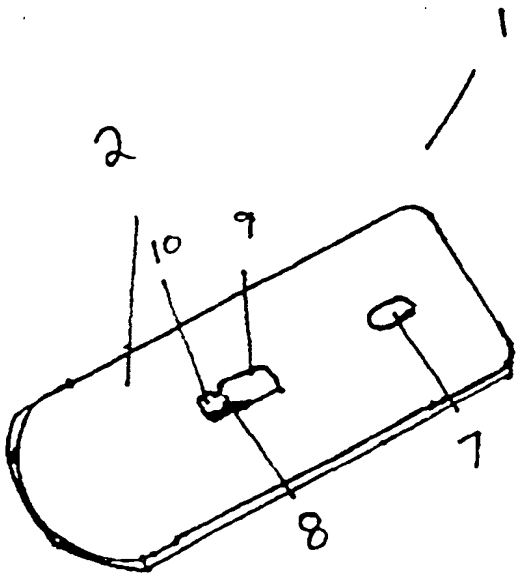

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Fig 2

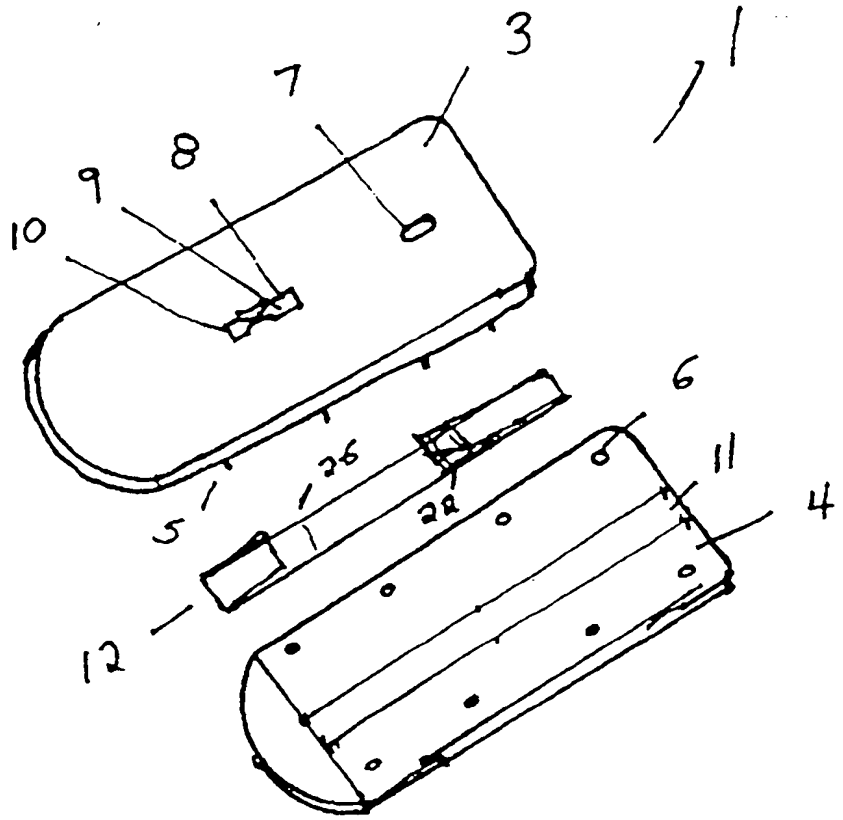
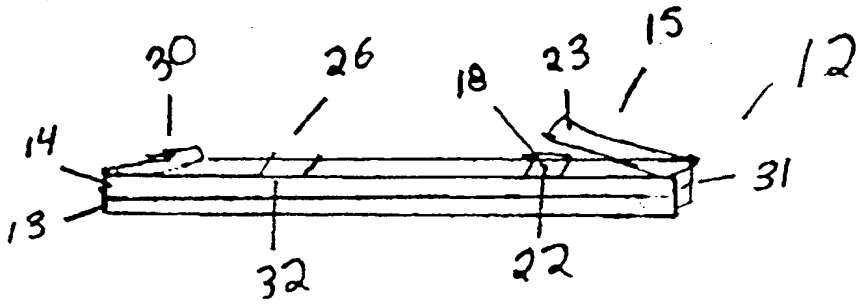


Fig 3



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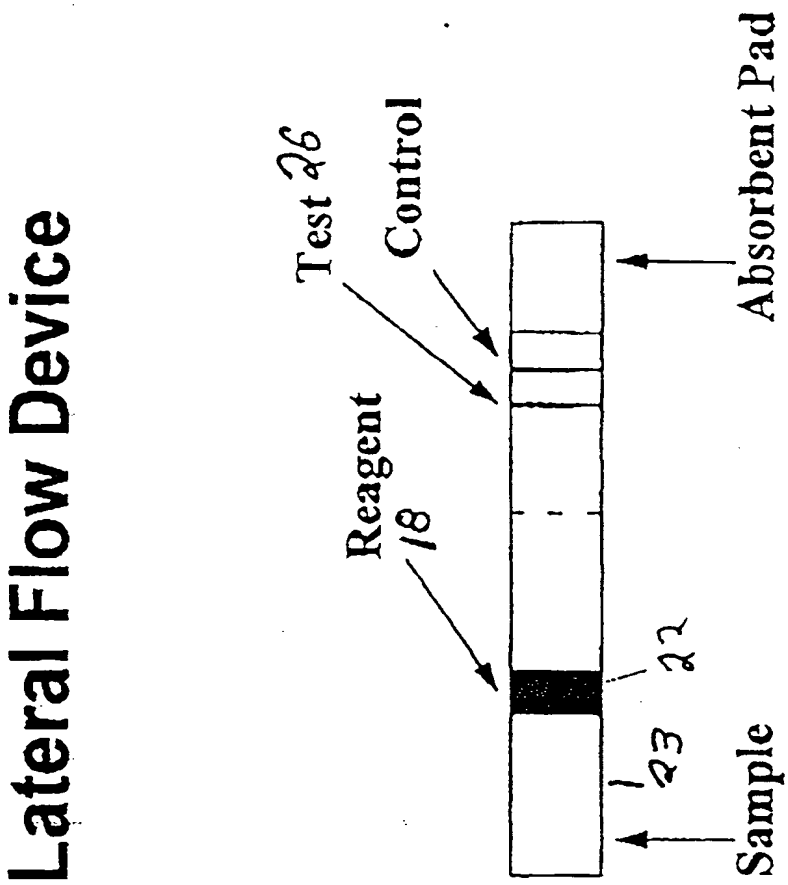

Fig. 4

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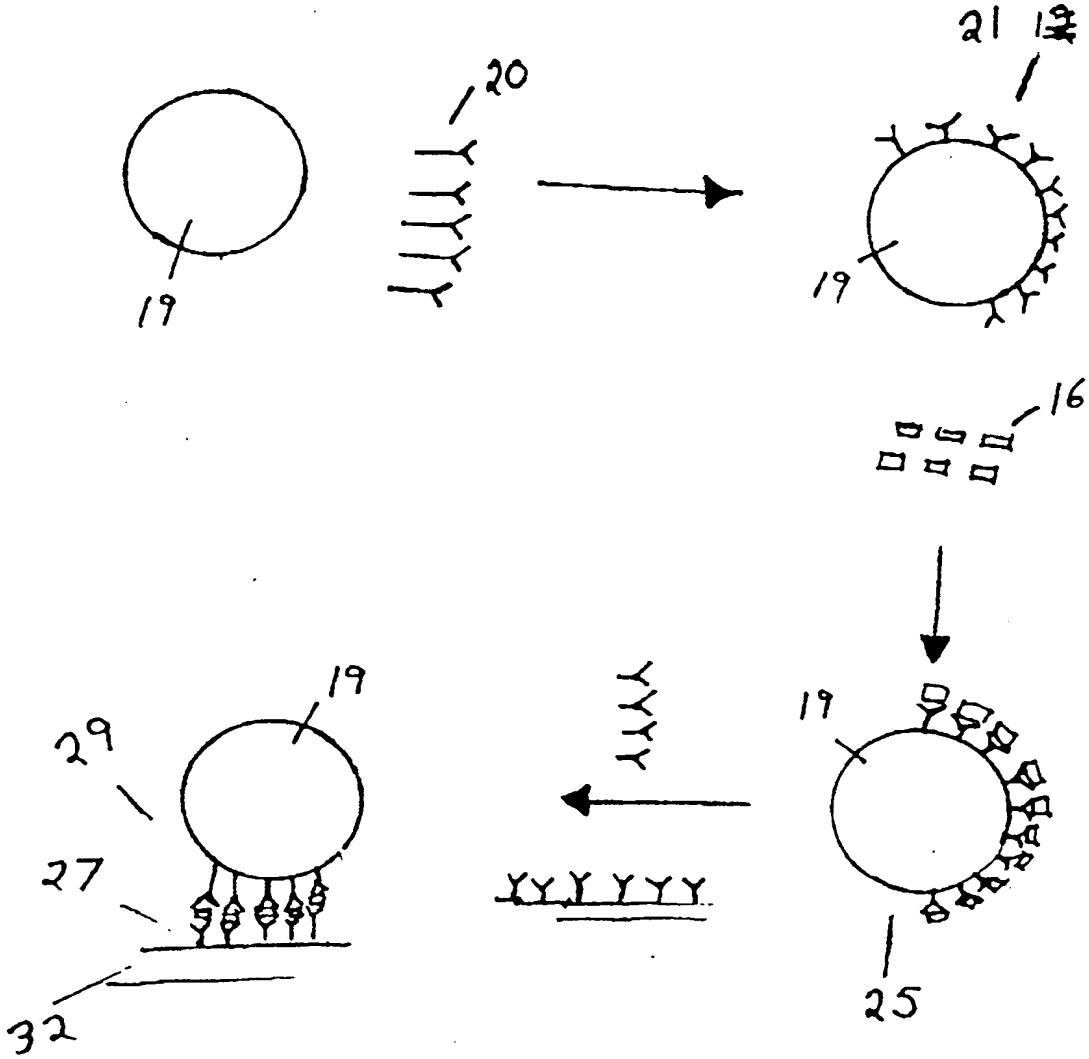

Fig. 5

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/518, 523, 524, 525, 528, 531, 535, 800; 422/56, 57, 58, 68.1, 69, 70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,571,667 A (CHU et al.) 05 November 1996, col. 3, lines 10-50.	1-6, 8-10
Y	US 5,514,602 A (BROOKS, JR. et al.) 07 May 1996, col. 3, line 41-col. 5, line 23.	1-6, 8-10
Y	US 5,500,350 A (BAKER et al.) 19 March 1996, col. 1, line 65-col.3, line 26.	1-6,8-10
Y	US 5,384,265 A (KIDWELL et al.) 24 January 1995, col. 4, line 45-col. 7, line 53.	1-6,8-10
Y	US 4,361,537 A (DEUTSCH et al.) 30 November 1982, col. 2, line 65-col.3, line 30.	1-6, 8-10
A	US 5,120,643 A (CHING et al.) 09 June 1992, entire patent.	1-6,8-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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Date of the actual completion of the international search
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,079,174 A (BUCK et al.) 07 January 1992, entire patent.	1-6, 8-10
A	US 5,604,110 A (BAKER et al.) 18 February 1997, entire patent.	1-6,8-10
A	US 5,372,931 A (FRIEDMAN et al.) 13 December 1994, entire patent.	1-6,8-10
A	US 5,294,369 A (SHIGEKAWA et al.) 15 March 1994, entire patent.	1-6, 8-10

RNSDOCID: <WO 9964863A1 1 >

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 7, 11 and 12
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

CLAIM 7 IS A DUPLICATE OF CLAIM 2.
CLAIMS 11 AND 12 DO NOT FURTHER LIMIT THE TEST SYSTEM BECAUSE THE LIMITATIONS RECITED IN THESE CLAIMS ARE NOT PART OF THE TEST SYSTEM.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 33/543, 33/544, 33/545, 33/546, 33/551, 33/553, 21/00, 31/22, 15/06, 33/00, 33/48, 30/96, 30/02

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US CL :

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CORRECTED VERSION*

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(54) Title: COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW ASSAYS UTILIZING SOLUBLE SUBMICRON PARTICLES

(57) Abstract

The present invention discloses a simplified, sensitive and specific immunoassay test procedure for the determination and detection of an immunologically reactive analyte in aqueous sample. A capture membrane comprising a dendrimer which aligns a capture antibody on a solid surface so that the capture capability of the capture antibody is optimized, resulting in a minimum loss in binding activity between the capture antibody and the ligand.

*(Referred to in PCT Gazette No. 11/2000, Section II)

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COLLOIDAL COLORIMETRIC FLOW THROUGH AND LATERAL FLOW ASSAYS UTILIZING SOLUBLE SUBMICRON PARTICLES

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention discloses a dendrimer-based, lateral flow, colorimetric assay test system.

2. Description of the Prior Art

The past twenty years have seen significant advances in rapid immunodiagnostic techniques. These methods include radio-imunoassay, counter electrophoresis, ELISA and membrane strip tests utilizing reagents tagged with a colorimetric label (e.g. gold, colored latex, etc.). Rapid diagnostic tests utilizing gold labeled antibody to visualize the test results on antibody coated membranes have evolved to be the test—of—choice in cases where manual, fast, and inexpensive, non—instrument test formats are required. The major area of concern, when utilizing the rapid gold test format, is the relative lack of sensitivity when compared to tests such as ELISA, which utilize enzyme enhanced reactions and prolonged multiple incubations steps and prolonged time.

Over the years, there have been many gold immunoassay test systems developed to show positive results in the face of particular antigens or antibodies. These assay systems utilize a colloidal gold particle concentration immunoassay to achieve sensitive and selective detection of biological materials. Antibodies specific to the agent of interest are conjugated to colloidal gold particles. Colloidal gold consists of discrete, electron-dense, red—colored particles ranging from 10 nm to 100 nm in diameter with a very high extinction coefficient. When concentrated on solid surfaces, these particles can be visually observed. Labeled antibodies can be easily dried and reconstituted without losing activity or specificity.

If antigen is present in a collected sample, an immune complex will form between the colloidal gold-labeled detector antibody (Ab) and the antigen (Ag). The test sample is used to reconstitute a dried colloidal gold labeled antibody and the resulting mixture (antibody and test antigen) ascends chromatographically up a strip that has been layered with a capture antibody, or directly through an antibody coated

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membrane. The presence of a red stripe or a dot is indicative of a positive test. The test strips contain a positive control to ascertain that the test is working properly.

U.S. Patent No. 5,514,602 (Brooks, Jr. et al.) discloses a method of producing a metal sol reagent containing colloidal metal particles. A metal containing solution is reduced under optimized pH conditions to produce metal sol particles of a preselected size. The particles are coated with a coupling compound, and then bound with at least one selected immunochemically reactive component. Particles having different immunochemical specificities are also mixed to produce reagents having multiple selected immunochemical specificities.

U.S. Patent No. 5,384,265 (Kidwell et al.) discloses contacting a sample which may contain an analyte with a biomolecule which is bound to a catalytically active colloidal metal particles, to obtain an analyte-biomolecule—colloidal metal particle complex, separating the analyte—biomolecule—colloidal metal particle complex from the sample, reacting the analyte—biomolecule—colloidal metal particle complex with hydrazine in the presence of lucigenin at a pH of 8 to 11; and detecting light generated by the reaction of the analyte—biomolecule—colloidal metal particle complex in the presence of lucigenin.

U.S. Patent No. 5,294,369 (Shigekawa et al.) discloses a gold sol coated with alkanethiols and alkanethiol derivatives which provide groups on the sol available for the linking of binding moieties such as antibodies, antigens or ligands to the gold sol.

U.S. Patent No. 5,334,538 (Parker et al.) discloses a gold sol immunoassay system and device. The gold sol bead is held in a funnel member. Antibodies are associated with the gold sol bead. When the sample contacts the gold sol, it dissolves the bead. A second antibody is impregnated on an immunosorbent surface. When the dissolved gold sol passes this surface, any antigen already reacted with the first antibody present reacts with the second antibody forming a gold:first antibody:antigen:second antibody: immunosorbent complex. The gold sol acts as the visible label.

U.S. Patent No. 5,120,643 (Ching et al.) discloses a process for immunochromatography with colloidal particles. The method comprises the steps of: contacting a chromatographic medium with the test sample, with the medium comprising at least two reaction sites. The first reaction site comprises a dried solution of a labeled specific binding reagent in the presence of a meta-soluble protein, and a

second reaction site comprising an immobilized specific binding reagent in relation to the presence or amount of the analyte in the test sample. The labeled reagent is solubilized and at least a portion of the labeled reagent is transported to the second reaction site, with the binding dependent upon the presence or amount of the analyte in the test sample. The labeled reagent is detected at the second site to determine the presence or amount of the analyte in the test sample.

U.S. Patent No. 5,079,172 (Hari et al.) discloses a method and kit for detecting the presence of antibodies using gold-labeled antibodies. Microspheres coated with an antigen reactive with the first antibody are reacted with the first antibody from serum or other sources. The gold-labeled antibody is reacted with the first antibody antigen complex on the microsphere and detected. Preferably, the gold particles are detected using an electron microscope.

PCT / US95 / 04547 describes the use of soluble submicron particles (dendrimers) that are labeled with antibodies to coat membranes. The assay procedures described are flow-through ELISA and fluorescence immunoassays requiring washing steps in order to obtain a response.

Currently, most lateral flow and flow-through gold immunoassays utilize antibody bound directly to porous membranes or to particles such as glass or latex to capture antigen-antibody-gold colored complexes.

The original tests developed by New Horizons Diagnostics utilized primarily 20 nm gold particles to tag the antibodies. This resulted in a sensitivity level of about 50 ng for botulism toxin and around 10^5 organisms/cc for the quantity of specific bacteria that could be detected. Particles, 20 nm in diameter, were used initially because of the stability of the gold tagged antibody conjugate and the low background signal (if any) it gave in negative test samples. Ideally, it is better to label with a much larger gold colloid (anywhere between 50 nm to 100 nm) to increase the sensitivity of test results. However, the problem with using larger colloidal gold complexes for tagging has been the increase in non-specificity, which creates false positive reactions.

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SUMMARY OF THE INVENTION

The present invention proposes a much more sensitive immunoassay test, which is easier to use and interpret. The entire test is conducted on a test strip and the detection antibody is preferably a FAB fragment that has been labeled with a 50-100nm gold particle and immobilized on a test pad. The capture antibody is bound to a soluble submicron particle (dendrimer) which is used to coat the membrane and become the capture site. The invention provides a simplified, sensitive and specific test procedure for the determination and detection of an immunologically reactive analyte in an aqueous sample.

Specifically, the present invention discloses the use of capture dendrimers to align and secure capture antibodies on a solid surface so that the immunological activity of the capture antibody is not sterically hindered. Consequently, optimal binding capacity is achieved, resulting in a minimum loss in binding activity between the capture antibody and the ligand.

In another preferred embodiment of the invention, the gold immunoassay test system uses larger gold colloids for tagging antibodies (anywhere between 50 nm to 100 nm) to increase the sensitivity of test results, (i.e. it is easier to detect larger particles because of the color intensity) without any residual non-specific activity. The elimination of non-specific background activity problems results from the use of blocking chemistries which inhibit non—specific reactions without altering the desired specific-reaction, the use of dendrimer bound antibody as the capture reagent, and the use of specifically designed recombinant FAB antibodies.

FAB antibodies are unlike whole antibodies in that their FC or constant regions are eliminated. It is this region of the antibody molecule that often causes problems with non—specificity.

In this invention, the FAB antibodies for detecting a positive result are attached to gold colloidal masses, in the range of 50-100 nm. These gold-FAB antibody complexes are positioned on a test strip, downstream from where the antigenic sample is applied.

Additionally, other metals or dyes may be attached to the FAB antibodies in place of the gold particles.

Further downstream from both the antigenic or ligand sample and the gold—FAB sample is a set of antibodies, specific for the gold-FAB antibody-antigen or antibody-ligand complex. These antibodies serve to concentrate the complex in one location, thereby allowing for a red stripe to appear on a set section of the test strip when there is a positive reaction.

The capture antibodies are located further downstream from the reagent and the sample site.

Dendrimers are one way of attaching the capture antibodies to the test strip. Dendrimers are three dimensional, tree-like polymers. The dendrimers have a small size, good solubility, high segmental density, interior void space, and low viscosity. Dendritic polymers can be constructed by both divergent and convergent synthetic methods. The divergent synthesis starts from a center core, and then grows each layer in a stepwise fashion, while the convergent method assembles exterior end groups and dendrons first before being coupled onto a core. Each re-iteration or layer is defined as a generation. The more layers there are in the dendrimeric structure, the more rigid the dendrimer molecule itself becomes. Up to about G8, a dense packing stage also occurs, where beyond this point, monomers can no longer react with every surface group quantitatively due to the stearic effect. In the case of immunoassays, the rigid, spherical protein—like sizes control the antibody binding direction, while the exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion onto a membrane surface. This property allows the dendrimer-antibody conjugate to self-assemble to its best binding orientation. As a result, the sensitivity and reproducibility of the immunoassay in the detection of different agents has been significantly improved, while the assay time has been shortened. In addition due to the nature of its low viscosity and self assembling capability of the dendrimer-antibody conjugates, the process for producing these tickets has been simplified, and has eliminated lot to lot variability.

Dendrimeters have the advantage that they can be synthesized with an exact uniform molecular weight, whereas conventional polymers always have a particular molecular weight distribution. In addition, dendrimers with particular functional groups can be manufactured with a defined number of such reactive groups.

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The coupling of antibodies to outer surfaces of dendrimers (for example polyamidoamine [PAMAMS] dendrimers) can be done by various well known chemistries which describe carbon sulfur, carbon-oxygen, and carbon-nitrogen coupling procedures. The exterior reactive surface groups are the key for linking dendrimers covalently with antibodies as well as providing adhesion onto the surface of a membrane. The structural composition of the dendrimer controls the spatial arrangement of the attached antibody molecules. This assures the optimal binding activity of the immobilized capture antibody.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects, features and advantages of the present invention will become more readily apparent from the following description, reference being made to the accompanying drawings in which:

FIG. 1 is an overall view of the immunoassay test system;

FIG. 2 is an exploded view of the immunoassay test system;

FIG. 3 is a side view of the immunoassay test strip;

FIG. 4 is an overhead view of the immunoassay test strip;

Fig. 5 is a schematic drawing of the antigen-antibody reaction.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

Referring to Figures 1—5, the immunoassay test system 1 comprises an enclosure 2, which is preferably plastic. This plastic enclosure comprises a top section 3 and a bottom section 4 which are held together by male 5 and female 6 peg joints.

The top section 3 of the enclosure 2 has an opening 7 for placing a sample. There is also an opening 8 to visualize the test results 9 and the control results 10.

The bottom section 4 comprises a tray 11 into which fits a test trip 12. The test strip 12 preferably has a membrane support 13. The membrane support 13 may be comprised of plastic, cardboard, or any other rigid material. On top of the membrane support 13 is a testing layer 14, preferably made out of nitrocellulose. On top of the nitrocellulose or testing layer 14 are the areas to which the appropriate reagents or samples are applied or affixed. The nitrocellulose/testing layer is affixed to the membrane support 13 by an adhesive 31.

At one end of the test strip 12 is the sample site 15 to which the sample is to be applied. This sample site 15 may have another nitrocellulose or adsorbent pad 23 residing on top of the testing layer, to which the sample is transferred. The sample may be in the form of an antigen or ligand 16 in a fluid.

The list of target ligands and anti-ligands which potentially may be detected includes antigens and ligands as well as antigens associated with bacteria, parasites, fungi, viruses, toxins, etc. Moreover, therapeutic drugs and controlled substances having small molecules, such as, for example, theophylline, may be detected or determined using the present invention.

The sample travels downstream from the sample site 15 to the gold immunoassay site 18 where LAB antibody-coated gold sol particles 19 reside. The gold particles 19 attached to the FAB antibodies 20 are preferably larger than 20 nm, more preferably in the range of about 50 to 100 nm, and most preferably in the range of from about 70 to 90 nm. Larger particles may also be used wherein a number of FAB antibodies 20 are attached to the gold particle 19. The gold sol labeled FAB antibodies 21 are preferrably dried and deposited on the strip 12.

The metal sol particles to be used in accordance with the present invention may be prepared by coupling an immunologically reactive substance directly to the gold particle. Additionally, the labeled component may be prepared by coupling the substance to the particle using a biotin/avidin linkage. In this latter regard, the substance may be biotinylated and the metal containing particle coated with an avidin compound. The biotin on the substance may then be reacted with the avidin compound on the particle to couple the substance and the particle together. In another alternative form of the invention, the labeled component may be prepared by coupling the substance, such as bovine serum albumin (BSA), and using this antibody as a carrier to bind to the metal particle.

The metal sol particles to be used in accordance with the present invention may be prepared by methodology which is well known. For instance, the preparation of gold sol particles is disclosed in an article by G. Frens, *Nature*, 241, 20-22 (1973). Additionally, the metal sol particles may be metal or metal compounds or polymer nuclei coated with metals or metal compounds, as described in U.S. Pat No. 4,313,734. Other methods well known in the art may be used to attach the gold particles to the FAB

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antibodies. The metal sol particles may be made of platinum, gold, silver, or copper or any number of metal compounds which exhibit characteristic colors and high extinction coefficients.

Similarly, the antibodies do not necessarily have to be attached to a metal sol particle, but may instead be attached to a dye with an extinction coefficient equal to or greater than gold.

There are a number of ways in which the gold labeled FAB antibodies 21 may be deposited on the strip 12. The gold labeled FAB antibodies 21 may be lyophilized or dried on the strip.

In the preferred embodiment, the gold labeled FAB antibodies are deposited and dried on a rectangular adsorbent pad 22, preferably about .25" x .25" or less. This FAB antibody pad 22 is positioned downstream from where the sample is applied on the strip 12. Preferably, the FAB antibody pad 22 fits underneath the distal end 24 of the sample pad 23.

If the sample contains an antigen or ligand 16 to which the gold FAB antibodies 21 react, there is an antigen-antibody bonding between the sample and the gold FAB antibodies 21. The antigen-gold FAB antibody complex 25 continues to migrate along the nitrocellulose strip 12 to the capture site 26 where the dendrimer bound antibody is fixed 12.

The antibodies 27 supported by the dendrimers 32 are designed to react specifically with the antigen, effectively forming an antibody-antigen-gold FAB antibody sandwich 29 if there is a positive reaction. If there is a negative reaction, no "sandwich" is formed, and the unreacted labeled antibody proceeds to the end of the strip 12 wherein an absorbent pad 30 absorbs the fluid and unreacted ligand that has migrated to the end of the strip 12.

To prepare the capture site 26, the antibodies are chemically bound to the dendrimers prior to their placement on the strip 12. This is then layered on the nitrocellulose strip and dried.

In performing the testing of an antigen or ligand, it should be noted that if there is a positive test result, the antigens or liqands will attach to the gold FAB antibodies as they migrate from the sample site, whereupon the antigens or ligands will attach to the

antibodies attached to the dendrimers. At this point, the concentrated gold particles appear as a red to purple line. If, however, the ligands do not attach to the gold labeled antibodies, the antibodies will not be bound by the capture site 26.

The superior sensitivity of this test format allows for detection of antigens or ligands in picogram quantities.

Additionally, this test can also have a positive control line. The positive control line has an anti-Fab substance or antibody laid down at the appropriate spot on the strip, downstream from the sample test site and from the FAB reagent site. This line should always appear when FAB antibodies are used in the test. If the positive control is negative, then the test is invalid.

Most immunoassay procedures are conducted at essentially neutral pH (7.5 to 8.0). In the preferred embodiment of this invention, the pH is elevated from pH 9 to pH 12. This is necessary to ensure that the positive surface charge on the dendrimer—antibody complex does not react non—specifically with the FAB-Gold labeled antibody and therefore yield false positive results.

EXAMPLES:

LAB antibody specific to Botulism toxin A at a concentration of 1.45 mg/cc was labeled with colloidal gold approximately 70 nm in size, at pH 9.5 at a final concentration of 10 μg/ml of 70nm gold. The adsorption was completed overnight and the gold antibody conjugate was centrifuged removing any unlabeled antibody.

The gold FAB complex was placed in borate buffer with 0.1% non-IDET detergent and 15 μl was spotted onto a precut polyester membrane and dried in the oven at 45° c for one hour.

FAB antibody (anti Bot. tox) was chemically bound to polyamidoamine dendrimers at a concentration of about one antibody molecule per branched dendrimer site. The soluble dendrimer antibody conjugate was used to stripe a nitrocellulose chromatography strip.

Goat anti-Mouse FAB fragment was used as a positive control and it was striped in an area in close proximity to the test zone.

Another test strip was prepared as above with the following difference: (1) the gold labeled antibody was tagged with 20 nm gold. (2) the FAR anti-Bot. tox was

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layered directly onto the nitro cellulose membrane. Dilutions were made of Botulism toxin A. The antigen was placed in 200 μ1 of a potassium carbonate solution, pH 11.0. The test solution was dispensed into the well of a test device described herein to which the test strip was added.

RESULTS	200 ng	100 ng	50 ng	10 ng	1 ng	0.5 ng
70 nm gold-antibody-dendrimer Capture	+	+	+	+	+	+
20 nm gold-straight capture	+	+	+	—	—	—

The buffers and BSA were run as negative controls. There were no false positive reactions.

Many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood within the scope of the appended claims that the invention may be protected otherwise than as specifically described.

What is claimed is:

1) A colorimetric immunoassay test system, comprising:

a test strip which allows for fluid absorption and migration; said test strip comprising:

a testing layer, said testing layer comprised of an absorbent material;

a sample site at one end of the testing layer, to which a ligand is applied;

a reagent—colorimetric imnunoassay site, said reagent-colorimetric immunoassay site comprising a plurality of antibodies attached to colorimetric particles thereby forming colorimetric labeled antibodies, said colorimetric labeled antibodies residing at said reagent—colorimetric immunoassay site;

a capture site, said capture site comprising dendrimers and capture antibody, forming dendrimer-capture antibody complexes to retain ligand and ligand-colorimetric labeled antibody complexes;

wherein said ligand, when placed on said sample site, migrates to the reagent—colorimetric-immunoassay site, and then migrates to the capture site comprising said dendrimers, such that if said ligand reacts positively with the colorimetric labeled antibodies, said ligand—colorimetric labeled antibody complexes migrate to the capture site, and a positive response will appear as a colorimetric line at the capture site when a capture antibody— ligand—colorimetric labeled antibody sandwich is formed.

2) The immunoassay test system of claim 1, wherein said antibodies at said reagent-colorimerric immunoassay site are FAB antibodies.

3) The immunoassay test system of claim 1, wherein said colorimetric particles are selected from the group consisting of gold sol particles, silver particles, platinum particles, copper particles and encapsulated dyes.

4) The immunoassay test system of claim 3, wherein the colorimetric particles are gold sol particles.

5) The immunoassay test system of claim 4, wherein said gold sol particles may range in size from about 20 nm to about 100 nm.

6) The immunoassay test system of claim 5, wherein said gold sol particles may range in size from about 50 nm to about 100 nm.

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7) The immunoassay test system of claim 1, wherein said antibodies at the reagent-colorimetric immunoassay site are FAB antibodies.

8) The immunoassay test system of claim 1, wherein said ligand is an antigen.

9) The immunoassay test system of claim 1, wherein the ligands and anti-ligands which may be detected are selected from the group consisting of antigens and ligands associated with bacteria, parasites, fungi, viruses, toxins, therapeutic drugs, and controlled substances having small molecules.

10) The immunoassay test system of claim 1, wherein said dendrimers for aligning said capture antibodies are starburst dendrimers.

11) The immunoassay test system of claim 1 wherein the test is performed at a pH between about 8 to about pH 12.

12) The immunoassay test system of claim 11, where the test is performed is at a pH betweeb pH 9.5 to about pH 11.5

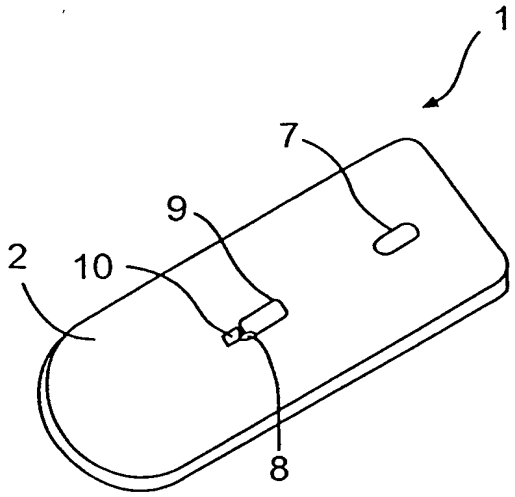

FIG. 1

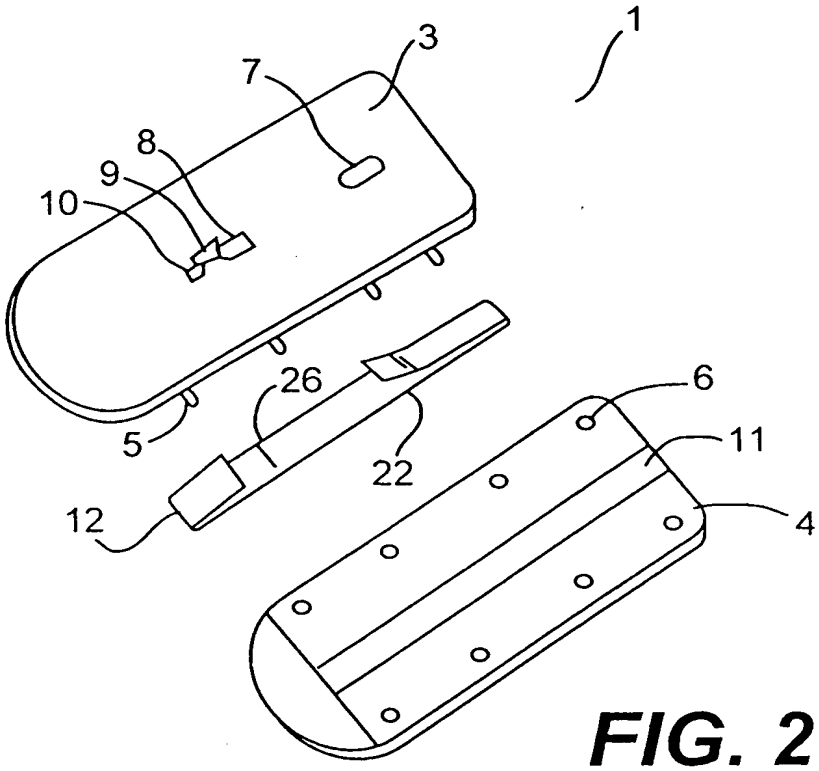

FIG. 2

BNSDOCID: <WO 9964863A1 IA>

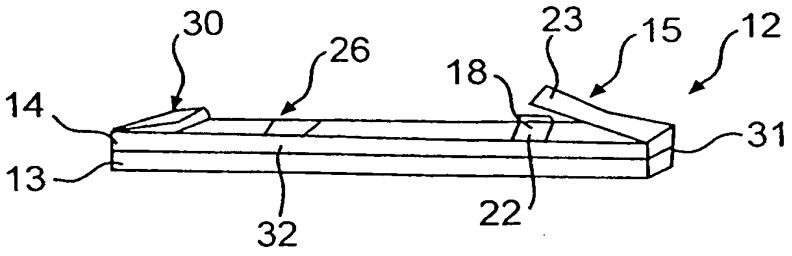
30
26
23
15
12
18
14
31
13
32
22

FIG. 3

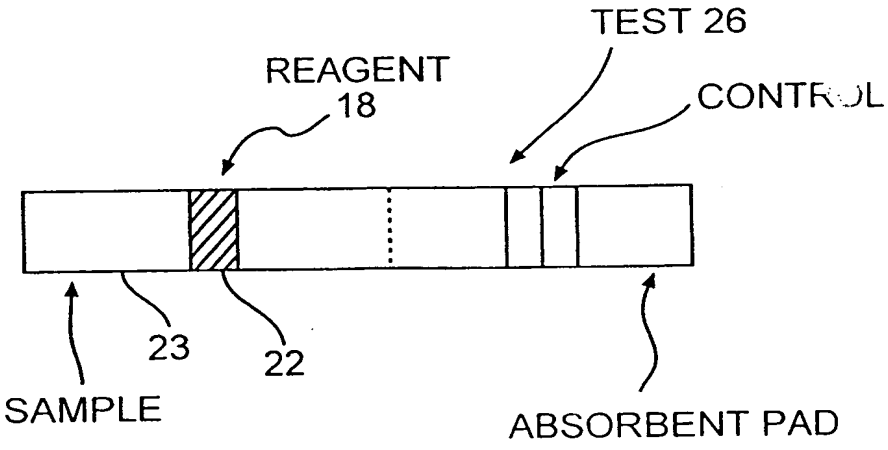
TEST 26
REAGENT
18
CONTROL
23
22
SAMPLE
ABSORBENT PAD

FIG. 4

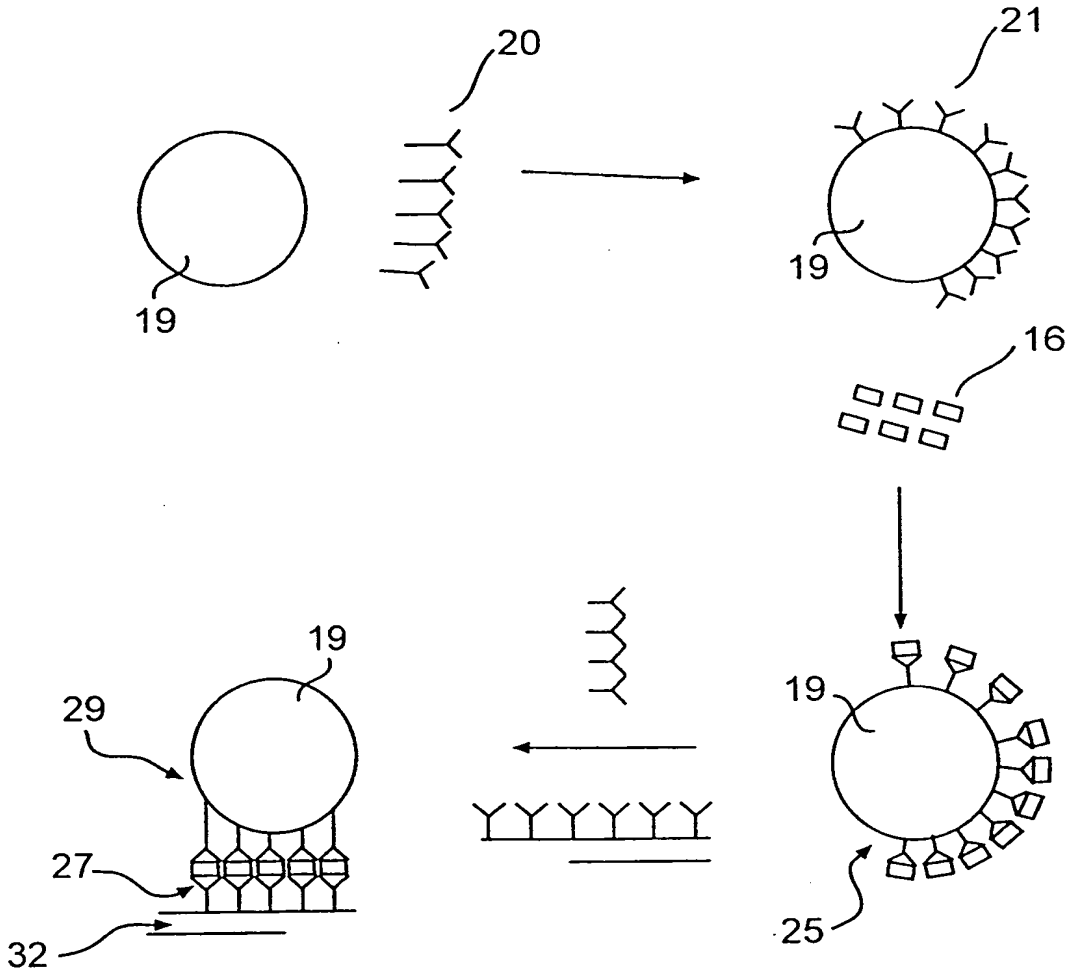

FIG. 5

BNSDOCID: <WO 9964863A1 IA>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/518, 523, 524, 525, 528, 531, 535, 800; 422/56, 57, 58, 68.1, 69, 70

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,571,667 A (CHU et al.) 05 November 1996, col. 3, lines 10-50.	1-6, 8-10
Y	US 5,514,602 A (BROOKS, JR. et al.) 07 May 1996, col. 3, line 41-col. 5, line 23.	1-6, 8-10
Y	US 5,500,350 A (BAKER et al.) 19 March 1996, col. 1, line 65-col.3, line 26.	1-6,8-10
Y	US 5,384,265 A (KIDWELL et al.) 24 January 1995, col. 4, line 45-col. 7, line 53.	1-6,8-10
Y	US 4,361,537 A (DEUTSCH et al.) 30 November 1982, col. 2, line 65-col.3, line 30.	1-6, 8-10
A	US 5,120,643 A (CHING et al.) 09 June 1992, entire patent.	1-6,8-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
04 AUGUST 1999

Date of mailing of the international search report
13 SEP 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,079,174 A (BUCK et al.) 07 January 1992, entire patent.	1-6, 8-10
A	US 5,604,110 A (BAKER et al.) 18 February 1997, entire patent.	1-6,8-10
A	US 5,372,931 A (FRIEDMAN et al.) 13 December 1994, entire patent.	1-6,8-10
A	US 5,294,369 A (SHIGEKAWA et al.) 15 March 1994, entire patent.	1-6, 8-10

BNSDOCID: <WO 9964863A1 IA>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 7, 11 and 12
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

CLAIM 7 IS A DUPLICATE OF CLAIM 2.
CLAIMS 11 AND 12 DO NOT FURTHER LIMIT THE TEST SYSTEM BECAUSE THE LIMITATIONS RECITED IN THESE CLAIMS ARE NOT PART OF THE TEST SYSTEM.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

RNSDOCID: <WO 9964963A1 IA>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/13258

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

G01N 33/543, 33/544, 33/545, 33/546, 33/551, 33/553, 21/00, 31/22, 15/06, 33/00, 33/48, 30/96, 30/02

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/518, 523, 524, 525, 528, 531, 535, 800; 422/56, 57, 58, 68.1, 69, 70

BNSDOCID: <WO 9964863A1 IA>